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EXPERIMENTAL CONDITIONS IN THE STUDY OF THE PHYSIOLOGICAL ECOLOGY OF ACTIVATED SLUDGE

by

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INTRODUCTION

During the past decade, the people of this nation have become increasingly aware of and alarmed over the threat of a diminishing supply of usable water. Efficient and rapid reclamation of water for re-use is linked with the improvement of biological aerobic treatment methods to the point that they are capable of rapid renovation of enormous quantities of waste water. The most popular biological unit is the activated sludge system which employs a heterogeneous population of microorganisms to stabilize wastes. Many investigators have studied the activated sludge process with a pragmatic goal of improved design and treatment, but as van Gils states, few have been concerned with the fundamental nature of the purification process (1).

Gaudy and Gaudy (2) indicate one approach to this problem by stating, "The challenging aspect to researchers in this field is the necessity of uncovering facts of predictive value for natural populations rather than for a single culture." However, they add, "Ideally, pure culture studies may form useful tools for the investigations of heterogeneous populations."

The overall objectives of the research include; (a) isolation, identification, and propagation of bacteria in activated sludge from a conventional treatment plant, (b) use of these cultures individually and as mixtures in oxygen uptake studies to determine the role of cultures in the metabolism of monocarboxylic acids and (c) measurement of content of individual fatty acids in aeration tank influent.

Studies on the ability of pure cultures of activated sludge microorganisms to clarify sewage have been made by a number of investigators. Butterfield, et al., (3), Ruchhoft, et al., (4), and Buck and Keefer (5), employing zoogleal species isolated from activated sludge, found that BOD removal by pure cultures is similar to that attained with the mixed flora of whole sludge. However, other workers reported that no pure culture was able to clarify domestic wastes as efficiently as sewage-seeded sludge, although some species approach 90 per cent efficiency. When van Gils compared the oxidation of selected organic compounds by pure cultures and by whole sludge, he found that members of the genera Pseudomonas, Arthrobacter, Flavobacterium, and Zooglea give oxygen consumption curves similar to those of activated sludge (1). No work comparing whole sludge oxidation patterns with those of pure and mixed cultures, employing the ratios of microbial populations existing in an aeration tank has been reported. Knowledge of the effect of one species upon another species or group of species is fundamental to attaining the goal of the systems analyst, i.e., development of a picture of the activated sludge aeration tank environment as a predictable functioning ecosystem.

Current investigations on the physiological ecology of activated sludge microorganisms are an attempt to bridge the gap between the knowledge gained through pure culture studies and predictive application to complex heterogeneous population dynamics.

Since by definition an ecosystem is a network of delicately balanced interactions between physical, chemical and biological forces, any rational interpretation of ecological dynamics requires that experimental conditions in the laboratory simulate as closely as possible the environment of the

community under study, in this case, the microflora of the aeration tank. Therefore, special attention was given to experimental parameters. This is a report on the establishment of the experimental conditions to be used in the study of the physiological ecology of activated sludge.

The literature contains numerous studies of the microorganisms in activated sludge, particularly Zooglea ramigera or other floc-producing types. Butterfield (11), after failing to develop floc-producers by transplant from agar, used an aerated nutrient broth to propagate floc organisms isolated from sludge by a capillary pipette. Other researchers following the same general methodology were able to plate out floc-producing organisms on nutrient agar (12,13).

McKinney and Horwood (6) and McKinney and Weichlein (14) isolated five different floc-producing species by alternate propagation on nutrient broth and nutrient agar. These organisms were initially separated from sludge flocs by shaking 1:10 dilutions of activated sludge in a bottle of distilled water containing sterile beads. Dugan and Lundgren (15) propagated sewage organisms on proteose-peptone yeast extract broth, isolating Z. ramigera after 48 hr incubation at 28°C. Nutrient agar proved to be a less suitable medium for colonial development. Dias, et al., obtained successful isolation of activated sludge organisms by initial growth on sewage agar, followed by propagation on PPYE broth (7). Crabtree and McCoy (16), recommended arginine broth as the preferred medium for isolation of Z.ramigera.

EXPERIMENTAL RESULTS

Activated Sludge Source. In order for the ecological study to be broadly applicable to municipal waste treatment in general, the source of the activated sludge should be a treatment plant handling typical domestic wastes containing no exotic wastes, since the bacterial flora of sludge is a function of the waste being treated (8). The treatment plant at Franklin, Tennessee, satisfies this criterion. In a previous study, Lutin, et al., (9) found that activated sludge from this plant showed an oxidation pattern similar to activated sludges from domestic waste treatment plants of the Columbus, Ohio area (10). The Franklin plant treats 0.74 MGD of combined domestic sewage and laundry wastes. Average aeration tank detention time is 6 hours with a 90 per cent BOD removal efficiency. The mixed liquor sample was collected aseptically at a point 1/3 of the way through the aeration tank on the morning of the day that the Warburg run was set up and isolation procedures began. Each sample was packed in ice and transported to the laboratory within one hour of collection. Aeration tank mixed liquor (ASML) adjusted to a selected solids concentration was used in the Warburg experimentation .

Warburg Studies. The Warburg respirometer technique was used for the oxidation studies because of its similarity to the physical, chemical, and biological characteristics of the conventional aeration tank. The experimental technique consisted of exposure of activated sludge or pure and mixed

pure cultures of activated sludge microorganisms to selected organic substrates with oxygen uptake as a measure of utilization of test compounds. The shape of the uptake curve and the total oxygen uptake in 24 hr are the fundamental parameters for interpretation of the enzymatic characteristics.

Substrates. The saturated monocarboxylic acids were chosen as the first chemical family to be investigated since their presence in domestic sewage had been reported by Murtaugh and Bunch (17). These compounds are known to be intermediate breakdown products in the biochemical degradation of protein, fats and carbohydrates (18). Fatty acids have also been demonstrated in urine (19). The sodium salts were used rather than the fatty acids since at the pH (7.2) of Franklin aeration tank liquor, the acids would be present as the salts. It has also been observed that fatty acids in human feces are usually in the salt form (20).

Previous studies indicated that compounds of this family yield characteristic oxidation curves with whole sludge and pure cultures (21,22,23). Both adaptive and constitutive enzymes for fatty acids occur in microorganisms (24). At least three different enzymes initiate β-oxidation of fatty acids depending upon molecular chain length (18).

Choice of Buffer. Since simulation of aeration tank pH is essential in the Warburg experimentation, the pH must be maintained in a narrow range

of about pH 7.2. The effect of various concentrations of phosphate buffer upon pH during oxidation of selected fatty acid salts are shown in Table 1. The buffer system employed was a combination of mono- and dipotassium phosphate. Used in a ratio of 1:2, the system has a pH of 7.20. The buffer solutions were sterilized by filtration through a HA membrane (0.45μ pore). The buffer strength indicated is the buffer concentration in the Warburg flask following addition of 10.0 ml of buffer solution to 10.0 ml of activated sludge mixed liquor. At the start of the run, the pH in all flasks was 7.2 to 7.3. Growth data for activated sludge microorganisms indicate that a pH of 7.5 is the upper limit of the optimum range (13,16). The experimental results indicated that with no buffer, the pH rose to 8 or above in virtually every case. A buffer strength of M/70 or stronger was capable of maintaining a pH of 7.5 or below in all flasks having an initial substrate concentration of 0.001M. The phosphate buffer systen contributes no extraneous carbon source and has wide application in studies of this nature.

In addition to maintenance of desired pH, there is a second consideration in the use of a phosphate buffer system. Jezeski, et al., (24) showed that bacterial oxidation of fatty acid salts was inhibited by M/6 phosphate buffer. Figures 1, 2, and 3 demonstrate that inhibition may occur in the presence of much weaker buffers. Figure 1 presents oxidation curves of activated sludge at 1500 mg/l suspended solids concentration, showing the effect of buffer in concentrations of M/62.5, M/83, and M/125,

TABLE 1. Influence of Various Concentrations of Phosphate Buffer upon Final pH at End of 24 hr Warburg Experiment.

Flask Contents	Final pH using following initial buffer concentrations (a)					
	None	M/60	M/70	M/85	M/100	M/125
(b) Sludge Control	8.21	7.30	7.30	7.35	7.37	7.41
Sodium Methanoate 0.001M	8.40	7.49	7.38	7.42	7.40	--
Sodium Methanoate 0.001M	8.54	--	--	--	--	--
Sodium Ethanoate 0.001M	8.60	7.37	7.40	7.40	7.45	--
Sodium Ethanoate 0.002M	8.60	--	--	--	--	--
Sodium Propanoate 0.001M	7.90	7.30	7.41	7.55	--	7.62
Sodium Propanoate 0.002M	8.00	7.45	7.50	--	--	7.70
Sodium Propanoate 0.003M	8.07	--	--	7.60	--	7.81
Sodium Butanoate 0.001M	8.60	--	7.42	7.46	--	--
Sodium Butanoate 0.002M	--	--	--	--	--	--
Sodium Pentanoate 0.001M	8.05	--	7.48	7.54	--	--
Sodium Octanoate 0.001M	8.23	7.40	7.42	7.54	7.60	--
Sodium Octanoate 0.002M	--	7.45	7.70	7.75	--	--
Sodium Dodecanoate 0.001M	8.00	7.40	7.42	7.51	7.52	--
Sodium Tetradecanoate 0.001M	8.05	7.30	7.43	--	--	--
Sodium Tetradecanoate 0.002M	--	7.41	--	--	--	--
Sodium Hexadecanoate 0.001M	--	--	7.30	--	--	--
Sodium Octadecanoate 0.001M	--	--	7.38	--	--	--
Sodium Octadecanoate 0.002M	--	7.38	7.51	--	--	--

(a) Buffer system was KH_2PO_4 + K_2HPO_4, ratio 1:2, pH = 7.20

(b) Franklin ASML, 1000 mg/1/

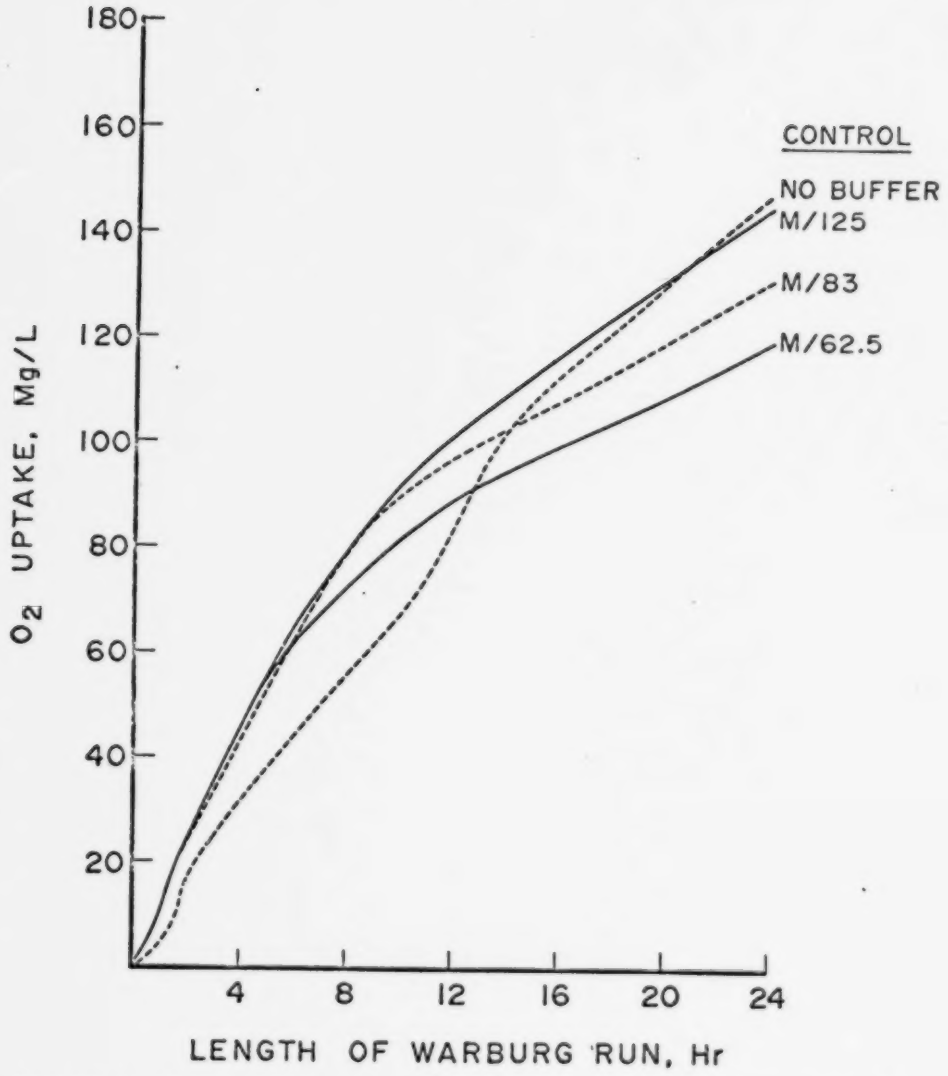

FIGURE 1. EFFECT OF VARIATION IN BUFFER CONCENTRATION UPON THE OXYGEN UPTAKE OF ACTIVATED SLUDGE (1500 mg/1) IN ABSENCE OF EXOGENOUS SUBSTRATE

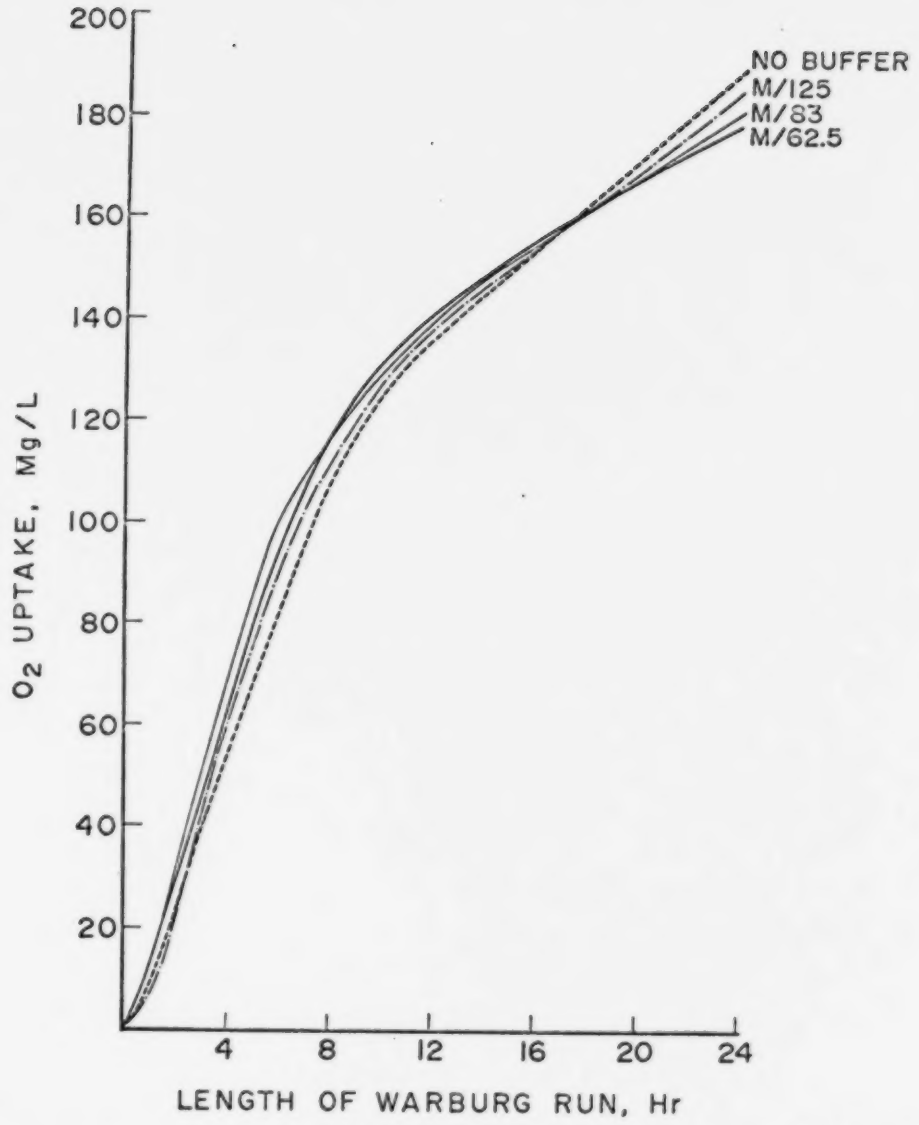

FIGURE 2. EFFECT OF VARIATION IN BUFFER CONCENTRATION UPON THE OXYGEN UPTAKE OF ACTIVATED SLUDGE (1500 mg/1) IN PRESENCE OF SODIUM PROPIONATE (0.001M)

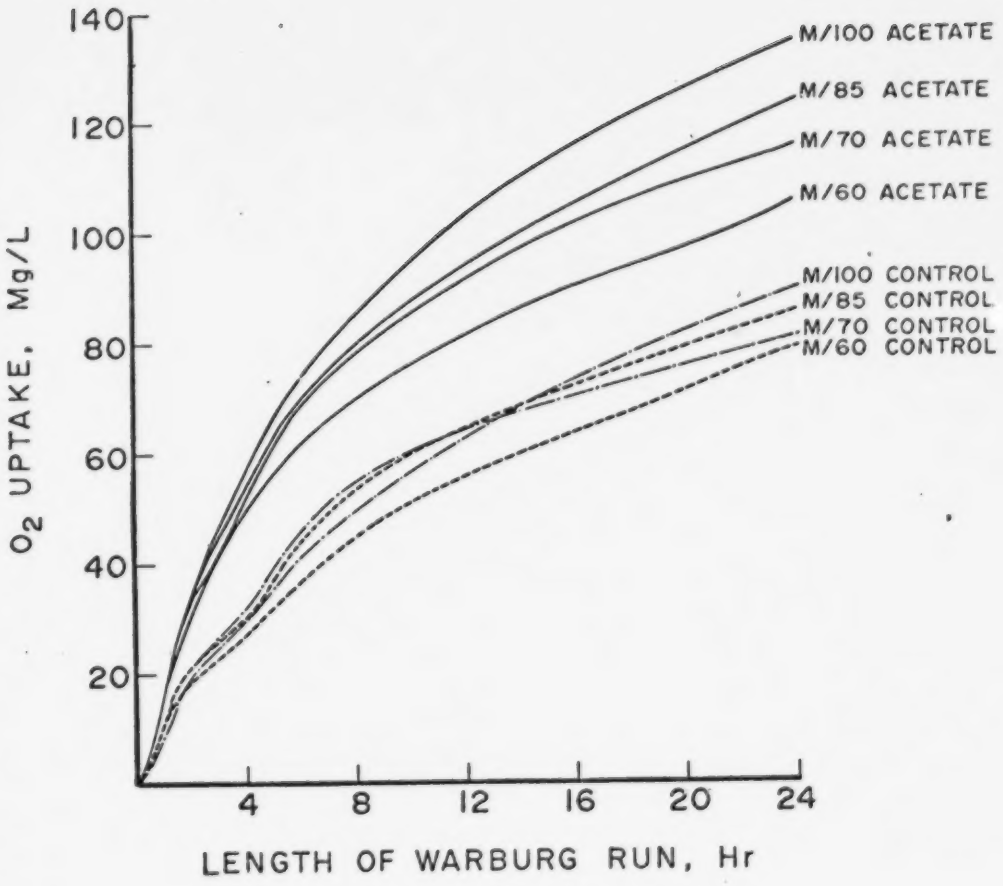

FIGURE 3. EFFECT OF VARIATION IN BUFFER CONCENTRATION UPON THE OXYGEN UPTAKE OF ACTIVATED SLUDGE (1000 mg/1) IN PRESENCE OF SODIUM ACETATE (0.001)

compared with a control flask containing distilled water instead of phosphate buffer. The control curve demonstrates a significant plateau-like lag while the buffer controlled curves are smooth and uniform, conforming to classic curves for constitutive enzyme action. The inhibition of total oxidation by increasing buffer concentration is obvious. This effect is not as pronounced in Figure 2, when 0.001M propionate is used as a substrate for activated sludge at 1500 mg/l suspended solids. The presence of the substrate appears to minimize the inhibitory effects of buffer use since the curves closely resemble one another. Another test (Figure 3) employing 0.001M acetate as substrate for 1000 mg/l sludge solids further illustrates the inhibitory effect of buffer concentration.

Since excessive buffer concentration may inhibit oxidation, the lowest buffer concentration compatible with maintenance of desired pH is most desirable. Therefore, the M/70 (0.014M) concentration was selected as optimum.

Substrate Concentration. The concentration of fatty acid salt in the Warburg flask was selected with due consideration to buffer strength and sludge suspended solids concentration. The molar basis of concentration was employed so that the reaction compartment of each Warburg flask contained the same number of molecules at the initiation of each Warburg run. A concentration of 0.001M was chosen since at that level oxidation by the sludge of the salt of lowest molecular weight (formate at 68 mg/l), would still have experimental significance when compared with oxygen uptake of

whole sludge (Figure 5), while, at the same time, the concentration of the longest salt used (i.e., myristate at 250 mg/l) would not exceed the limits of solubility to an excessive degree. At this concentration, myristate is not completely soluble but it can be seen in Figure 4 that this incomplete solubility does not affect oxidation, since total uptake for the 250 mg/l flask is roughly double that of the 110 mg/l flask where solution of the salt was complete. The diluent for substrate solutions was sterile M/35 phosphate buffer. The substrate was not sterilized. A 10/0 mg volume of substrate-buffer solution was added to each Warburg flask.

Suspended Solids Concentration. Dawes and Ribbons (25) reported that interpretation of endogenous respiration becomes more difficult when the rate of endogenous respiration is high as compared with the rate in the presence of exogenous substrate. This point was investigated in oxygen uptake runs at 1000 and 2000 mg/l solids with and without 0.001M formate as substrate (Figure 5). The curves deviate slightly during the first few hours, but at 24 hours the difference between control and formate uptake is essentially constant for all levels of solids concentration, suggesting strongly that this difference represents oxidation of formate. In this case, interpretation is easy.

This research will involve use of representative concentrations of pure culture organisms in Warburg oxidation studies. Monod (26) pointed out that the conditions in a culture medium vary in a largely uncontrollable and unknown way as the culture grows. These changes can be minimized by

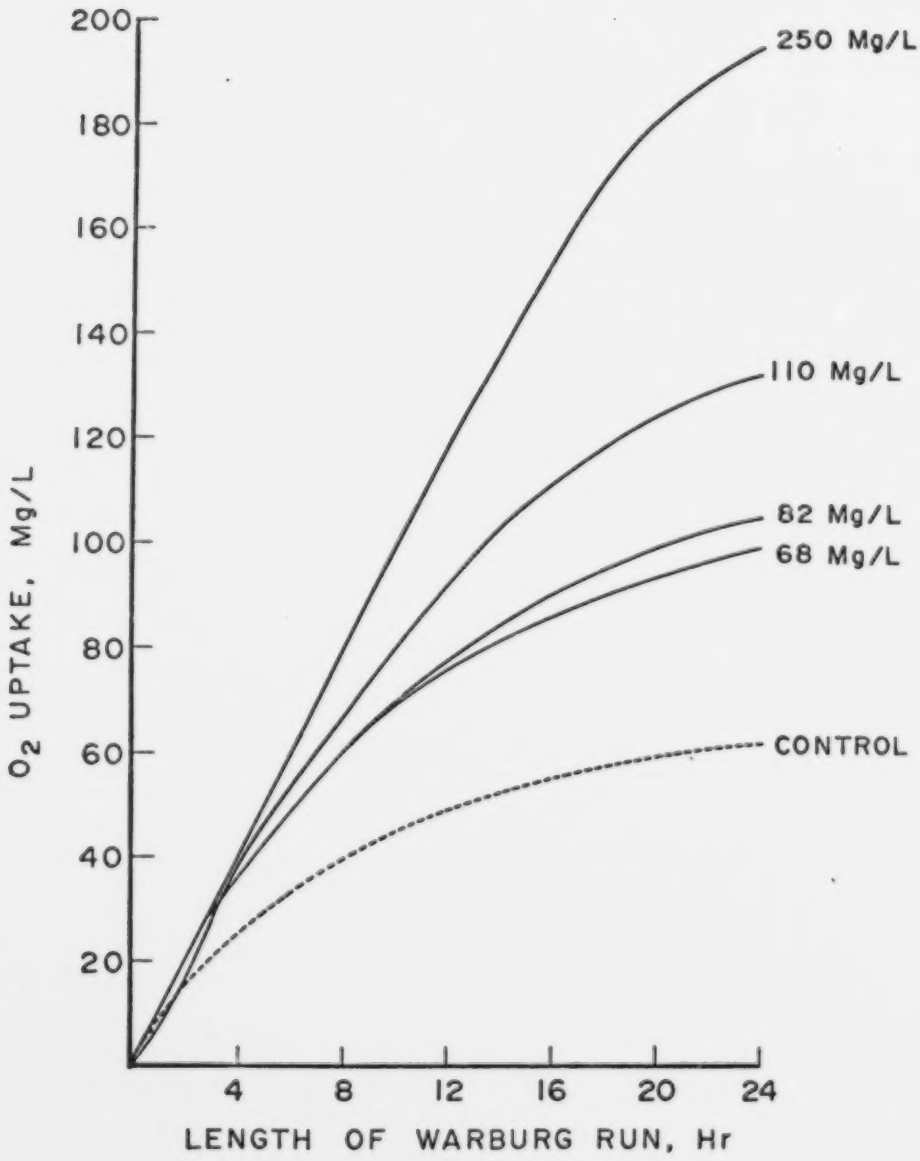

FIGURE 4. EFFECT OF VARIATION OF CONCENTRATION OF SODIUM MYRISTATE UPON THE OXYGEN UPTAKE OF ACTIVATED SLUDGE (1000 mg/l)

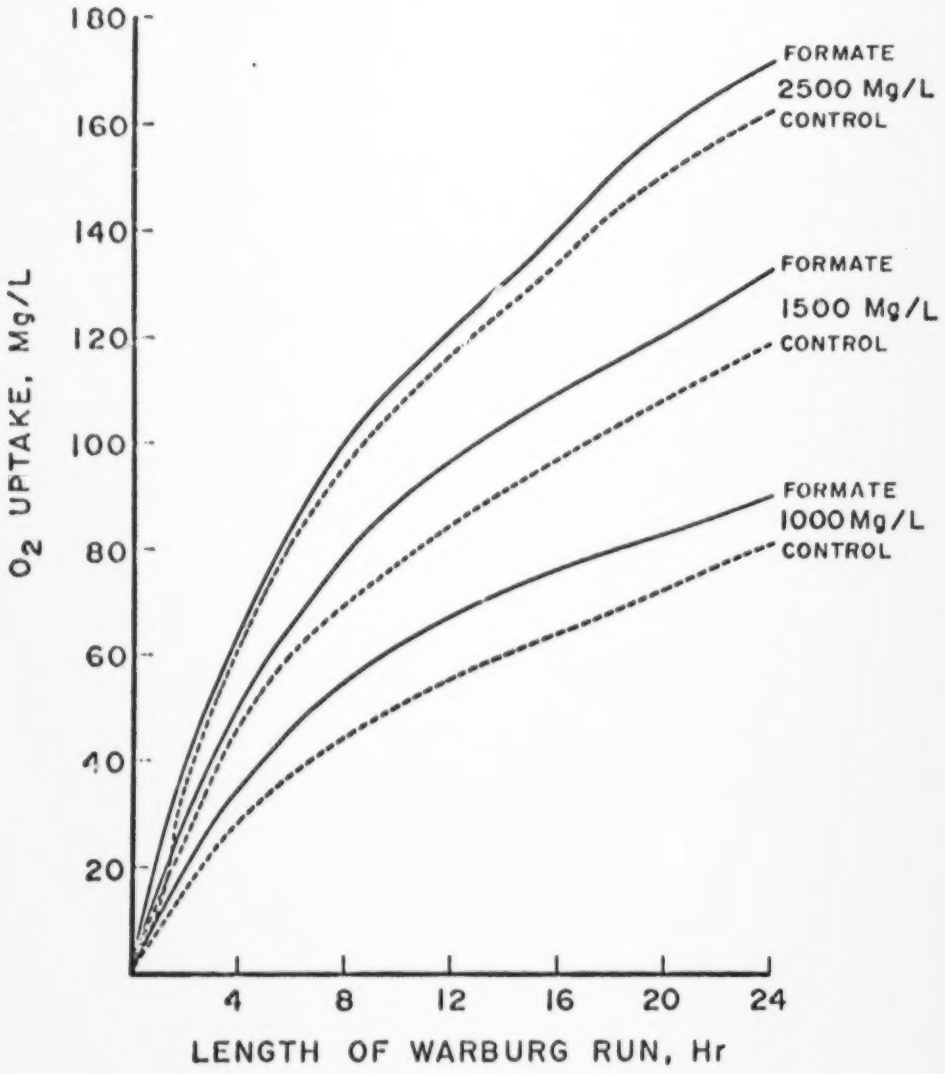

FIGURE 5. EFFECT OF VARIATION OF ACTIVATED SLUDGE SOLIDS CONCENTRATION UPON OXIDATION OF SODIUM FORMATE (0.001M)

use of dilute cultures. Therefore, the lower the solids concentration chosen for the Warburg experimentation, the more dilute the inoculum of pure culture can be. A third reason for choosing a flask solids concentration of 1000 mg/l was that the Franklin plant maintains an average aeration tank suspended solids concentration of 2100 mg/l. Therefore, only minor adjustment was necessary to obtain a suspension containing 2000 mg/l sludge. Any adjustment requiring dilution was achieved by addition of aeration tank liquor. While below usual operating concentration, 1000 mg/l suspended solids is within a reasonable range since concentrations down to 290 mg/l have been satisfactorily employed in activated sludge studies (6).

Autoxidation. Introduction of extraneous organisms into the reaction vessels by air contamination or substrate contamination might be a source of error. Autoxidation Warburg runs were carried out to check the possibility of such contamination. This test was made by setting up flasks containing 0.001M of each substrate in sterile buffer. The autoxidation flasks showed no oxygen uptake in 24 hr, indicating that introduction of extraneous organisms is of no experimental significance.

Washed Sludge vs Unwashed Activated Sludge. Washing of activated sludge theoretically removes extraneous nutrient material, as well as microorganisms not fixed to the floc. The latter organisms were assumed to be simply passers-by with no active role in purification.

To check the desirability of using washed sludge in the Warburg studies, parallel flasks were set up employing washed and unwashed (whole) activated sludge. Washing was accomplished by pouring 100 ml of ASML into a sterile graduated cylinder, allowing it to settle for 5 min and discarding the liquid portion by decantation. The volume was brought back to 100 ml by addition of sterile tap water and the cylinder gently inverted once. After a second settling and decanting, the washing procedure was repeated. The final suspension was adjusted to a solids concentration of 1000 mg/l. Figure 6 shows the effect of sludge washing on control and formate oxidation. The reduced total 24 hr oxygen uptake may be attributed to depletion of soluble nutrients by the washing process or to disturbance of the natural microenvironment. The curves in Figure 7 show net oxidation values (i.e., substrate uptake minus control uptake) using octanoate and formate as substrates. The divergent patterns taken by the octanoate curves in the first 2 hr and by the formate curves after 8 hr are indications of environmental disturbance or alterations which must be avoided in this study. For this reason, it was decided to use unwashed sludge in subsequent experimentation.

Reproducibility of Warburg Results. If information gained through a study of this nature is ultimately to be translated into improved waste treatment, the variability of conditions in a typical aeration tank must be known to some extent. In repeated Warburg runs over a period of three weeks in March, 1967, the oxidation curves for a selected group of test substrates were similar in nature (Figures 8 and 9) indicating a consistency of environment and enzymatic performance in the aeration tank of the Franklin plant.

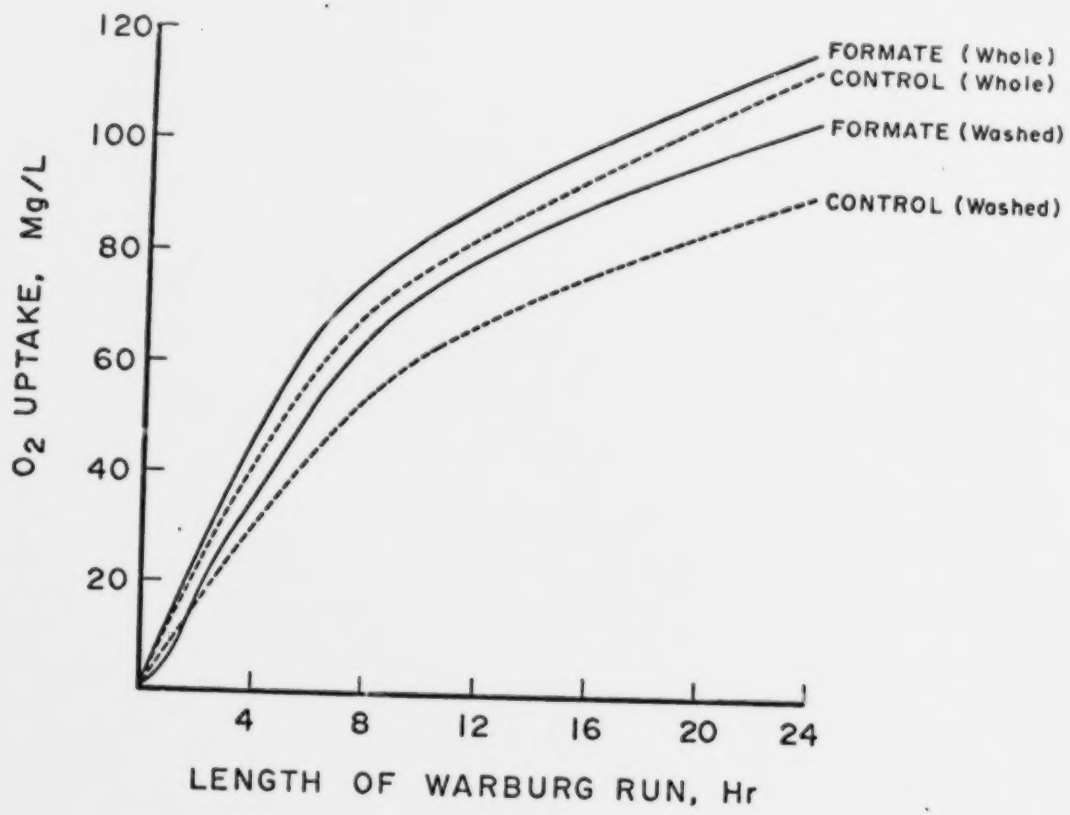

FIGURE 6. EFFECT OF SLUDGE WASHING UPON THE OXYGEN UPTAKE OF ACTIVATED SLUDGE (1000 mg/l) IN PRESENCE OF SODIUM FORMATE (0.001M)

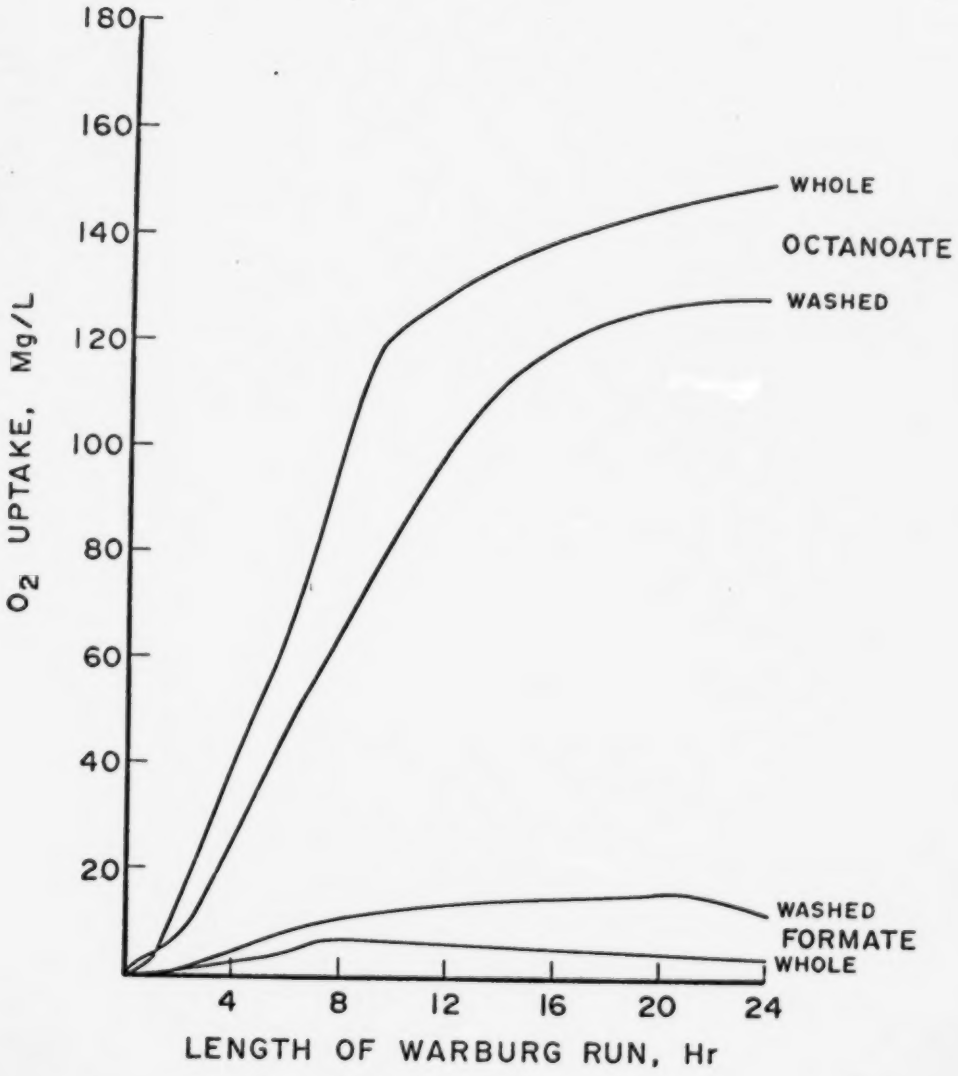

FIGURE 7. EFFECT OF SLUDGE WASHING UPON THE OXYGEN UPTAKE OF ACTIVATED SLUDGE (1000 mg/l) IN THE PRESENCE OF SODIUM FORMATE (0.001M) AND SODIUM OCTANOATE (0.001M)
(Values are corrected for control oxidation)

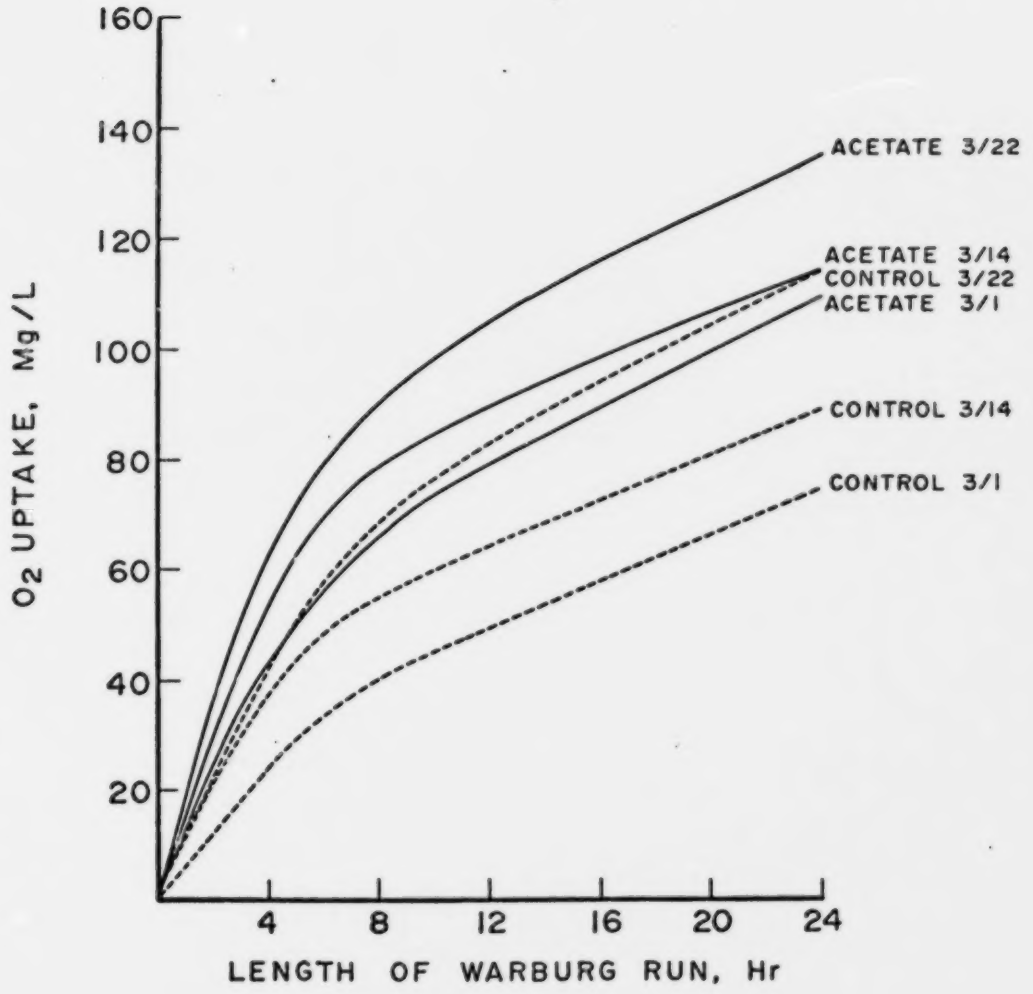

FIGURE 8. EFFECT OF THREE ACTIVATED SLUDGE SAMPLES (1000 mg/l) UPON THE OXYGEN UPTAKE IN THE PRESENCE OF SODIUM ACETATE (0.001M)

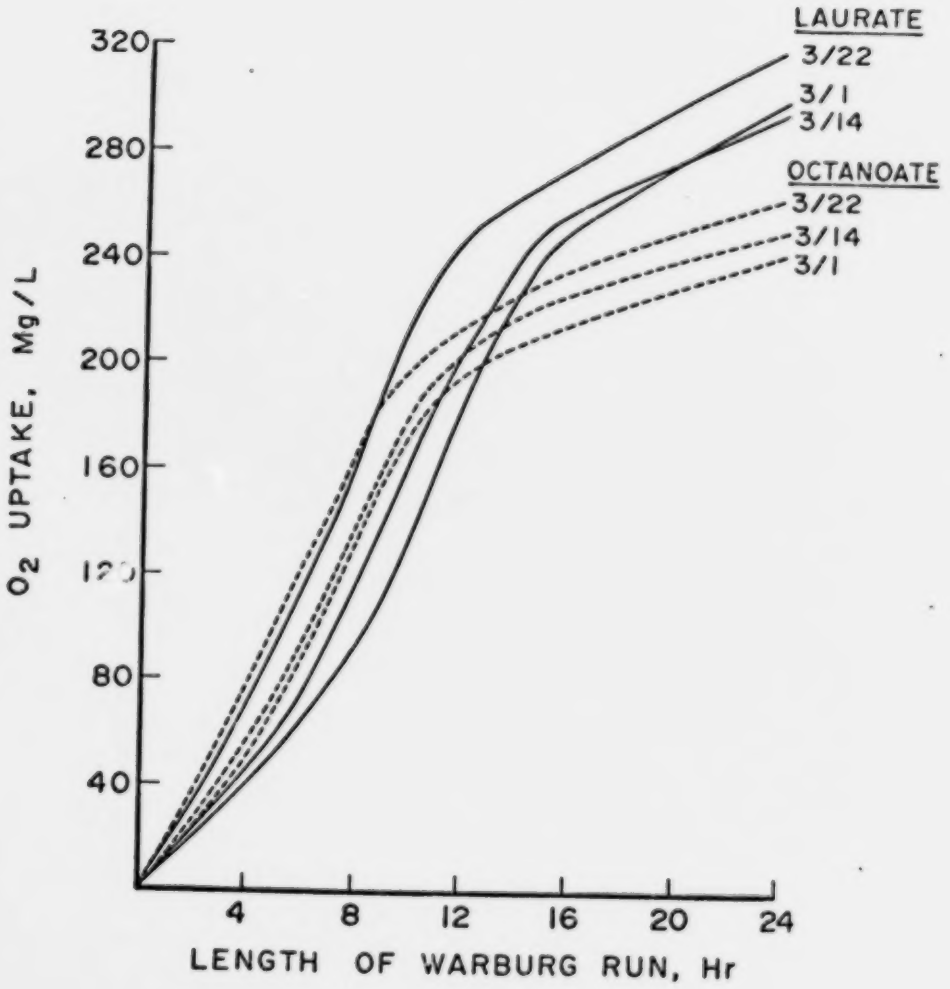

FIGURE 9. EFFECT OF THREE ACTIVATED SLUDGE SAMPLES (1000 mg/1) UPON THE OXYGEN UPTAKE IN THE PRESENCE OF SODIUM OCTANOATE (0.001M) AND SODIUM LAURATE (0.001M)

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